In vitro and *in vivo* examination of phosphate incorporation by *Escherichia coli* phosphorylase enzymes

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Declaration

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Abstract

Starch and glycogen are two storage polymers that are similar in structure, function and metabolism. Alterations in starch structure, including its phosphorylation, can alter its properties in a way that make it useful for industry. In this study the role of two *Escherichia coli* phosphorylase enzymes, glycogen phosphorylase (GlgP) and maltodextrin phosphorylase (MalP), in the incorporation of phosphate into glycogen is examined both *in vitro* and *in vivo*. *In vitro* analysis revealed that when MalP and GlgP were incubated with a primer and glucose-1,6-bisphosphate, glucose-6-phosphate was incorporated into glycogen. The K_m's of GlgP and MalP for glucose-1,6-bisphosphate were found respectively to be ~1.3 mM and ~0.7 mM when glycogen was used as a priming polymer, with a V_{max} of ~0.08 µmol/µg/min and ~0.05 µmol/µg/min respectively. To examine if this *in vitro* activity is relevant biologically, their *in vivo* role was explored using knockout mutants. *G*lycogen extracted from wild type *E. coli* and mutants lacking either MalP, GlgP or both, contained similar amounts of covalently bound phosphate indicating that it is incorporated by another mechanism.

Opsomming

Stysel en glikogeen is twee stoorpolimere met gelyksoorte struktuur, funksie en metabolisme. Veranderinge in die struktuur van stylsel, insluitend deur middel van fosforilering, kan sy eienskappe sodoende verander wat dit meer toepaslik maak vir industriële gebruike. In hierdie studie word die rol van twee *Escherichia coli* fosforileerings-ensieme, glikogeenfosforilase (GlgP) en maltodekstrienfosforilase (MalP), in die inkorporering van fosfaat in glikogeen *in vitro* en *in vivo* ondersoek. *In vitro* analise het getoon dat beide MalP en GlgP, in die teenwoordigheid van 'n inleier en glukose 1,6-bisfosfaat, die inkorporering van glukose 6-fosfaat in glikogeen kon uitvoer. Die K_m's van MalP en GlgP vir glukose 1,6-bisfosfaat is onderskeidelik gevind as ~0,7 mM en ~1,3 mM wanneer glikogeen as 'n inleier gebruik was, en die V_{max} gevind as ~0.08 µmol/µg/min en ~0.05 µmol/µg/min. Om te ondersoek of hierdie *in vitro* aktiwiteite biologies van toepassing is, was hul rolle *in vivo* ondersoek met behulp van mutante wat die betrokke gene ontbreek. Glikogeen geïsoleerdvanuit wild tipe *E. coli* en mutante ontbreek inMalP, GlgP of beide die gene, het soortgelyke hoeveelhede kovalent gebonde fosfaat bevat, wat dus daarop dui dat dit deur 'n ander meganisme geïnkorporeer word.

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ADP	adenosine diphosphate
Ag^{+}	silver ion
AMP	adenosine monophosphate
ATP	adenosine triphosphate
Cu^{2+}	copper ion
ddH_20	deionized distilled water
DNA	deoxyribonucleic acid
DP	degree of polymerization
DTT	dithiothreitol
E. coli	*Escherichia coli*
G1P	glucose-1-phosphate
G6P	glucose-6-phosphate
G6PDH	glucose-6-phosphate-dehydrogenase
GBP	glucose-1,6-bisphosphate
Glc	glucose
GlgP	glycogen phosphorylase
GST	glutathione-S-transferase
HCl	hydrochloric acid
Hg^{2+}	mercury ion
His	histidine
HK	hexokinase
I_2	iodine
IMAC	immobilized metal ion affinity chromatography

IPTG	isopropyl β-D-1-thiogalactopyranoside
K_2HPO_4	dipotassium hydrogen phosphate
Kan	kanamycin
KB	Kornberg
kDa	kilo-dalton
KH_2PO_4	potassium dihydrogen phosphate
KI	potassium iodide
KOH	potassium hydroxide
LB	Luria Bertani
M	molar
MalP	maltodextrin phosphorylase
mg	milligram
Mg^{2+}	magnesium ion
$MgCl_2$	magnesium chloride
mL	milliliter
mM	mill-molar
MOS	malto-oligosaccharides
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NaN_3	sodium azide
Ni-NTA	Nickel-nitrilotriacetic
nm	nanometres
PCR	polymerase chain reaction
PGM	phosphoglucomutase

P_i	inorganic phosphate
PMSF	phenylmethylsulfonyl fluoride
rpm	revolutions per minute
RT	room temperature
SBE	starch branching enzyme
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	standard error
SS	starch synthase
Tris	Tris(-hydroxymethyl)aminomethane
U	enzyme unit
UDP	uridine diphosphate
v/v	volume/volume
w/v	weight/volume
WT	wild type
Zn^{2+}	zinc ion
× g	gravitational acceleration
°C	degrees centigrade
µg	microgram
µl	microliter
µm	micrometre

Chapter 1: Literature review

1.1 Polymers

1.1.1 Structure and applications

Polymers are formed when similar subunits are covalently linked together. Bacteria produce polymers that are classified into four main classes: polyesters, polyamides, inorganic polyanhydrides, and polysaccharides (Rehm, 2010), many of which have uses in industry (Table 1.1). Polysaccharides produced by bacteria include intracellular polysaccharides, such as glycogen; exopolysaccharides, such as cellulose and dextran (which are synthesized or secreted extracellularly by enzymes that are anchored to the cell wall); and capsular polysaccharides, such as the K30 antigen (which are secreted, but remain attached to the cell) (Rehm, 2010). They can also be categorized according to their structure. Repeat unit polymers are formed when larger units are repeated to produce the polymer, such as xanthan, which is composed of repeated pentasaccharide units (Becker *et al.*, 1998; Whitfield, 2006). Repeating polymers are formed when a monomer is repeated to form the complete polymer, such as cellulose and glycogen, which are both made up of repeating glucose monomers (Valla *et al.*, 2009). Non-repeating polymers, such as alginate, do not have any specific monomer or unit that is repeated (Remminghorst and Rehm, 2006). Additionally, they can be classified as either homo-polysaccharides or hetero-polysaccharides. Homo-polysaccharides are formed when the same monosaccharides are linked together, whilst hetero-polysaccharides are composed of more than one kind of monosaccharide. Cellulose, starch, and glycogen are some of the most abundant homo-polysaccharides in nature and are composed of glucose. Many heteropolysaccharides provide extracellular support to organisms, including hyaluronic acid and chondroitin sulphates.

Table 1.1 Classification and industrial applications of bacterial polymers. Examples of the main bacterial polymer classes, their features, and uses (table adapted from Rehm, 2010).

Class	Structure	Localization	Main constituents	Industrial applications
Polyester				
Polyhydroxy-alkanoates	Heteropolymer	Intracellular	(R)-3-hydroxy fatty acids	Bioplastics; biomaterial; matrices for protein binding
Polyamides				
Cyanophycin granule peptide	Repeating heteropolymer	Intracellular	Aspartate and arginine	Dispersant; water softener
Polyanhydrides				
Polyphosphate	Homopolymer	Intracellular	Phosphate	Flavor enhancer; ATP replacement in enzymatic synthesis
Polysaccharides				
Homopolysaccharides				
Glycogen	α-1,6-branched α-1,4-linked homopolymer	Intracellular	Glucose	Not applicable
Cellulose	β-1,4-linked homopolymer	Extracellular	D-glucose	Wound dressing; diaphragms of acoustic transducers; food (nata de coco)
Dextran	α-1,2/α-1,3/α-1,4-branched α-1,6-linked homopolymer	Extracellular	Glucose	Blood plasma extender; chromatography media
Heteropolysaccharides				
Alginate	β-1,4-linked non-repeating heteropolymer	Extracellular	Mannuronic acid and guluronic acid	Biomaterial (e.g. for drug delivery or as a tissue scaffold)
Xanthan	β-1,4-linked repeating heteropolymer	Extracellular	Glucose, mannose, and glucuronate	Food additive (e.g. emulsifier or thickener)
K30 antigen	β-1,2-linked repeating heteropolymer	Capsular	Mannose, galactose, and glucuronate	Not applicable
Hyaluronic acid	β-1,4-linked repeating hereropolymer	Extracellular	Glucuronate and *N*-acetyl glucosamine	Cosmetics; tissue repair; drug delivery; viscosupplementation

1.1.2 Biological function

Organisms produce polymers to perform a variety of functions, including storage of nutrients and reserves, or for structural or protective roles (Rehm, 2010). Cellulose is the most common structural polysaccharide in nature. It is a linear polymer composed of glucose residues linked by β-1,4-O-glycosidic bonds. Long cellulose fibers are held together by intermolecular and intramolecular hydrogen bonds that occur within the same plane, as well as those above and below. The resulting fibrous bundles are well-suited for their structural role, and are not easily degraded (Teramoto, 2015). Another function of polymers is to store nutrients, such as carbohydrates and proteins. Carbohydrates are vital energy sources for many organisms and are commonly stored in the form of glucose polymers (Damager *et al.*, 2010). Glucose is primarily stored in the form of glycogen bodies in bacteria and animals (Manners, 1991), whilst plants generally store it as starch granules (Zeeman *et al.*, 2010). These two polyglucans are structurally similar, as both starch and glycogen are composed of glucose monomers linked by α-1,4-glycosidic bonds, with glycogen and amylopectin both having branchpoints linked by α-1,6 bonds.

This study examines an aspect of glycogen metabolism for two reasons. Firstly, to help to understand a newly identified process in *E. coli* glycogen, specifically glycogen phosphorylation (See section 1.3.2). Secondly to utilize this knowledge to improve the industrially important plant storage polymer starch, to include increased amounts of covalently bound phosphate. The next sections will, therefore examine the polymers starch and glycogen.

1.2 Starch

Starch is a storage polyglucan synthesized by plants that forms large (with diameters ranging from 0.1 μm to over 50 μm), insoluble granules within plastids (Ball and Morell, 2003). The polymer is composed of two fractions, one linear and the other branched named, respectively, amylose and amylopectin. The organization within the granules of these two components leads to the formation of insoluble semi-crystalline structures (Smith *et al.*, 1999). Amylopectin is the major component of starch (normally about 70% of the total) and is made up of α-1,4 linear chains that are linked together by α-1,6 bonds (Ball and Morell, 2003). It is structurally similar to glycogen, as both are composed of glucose moieties linked by the same type of bonds (Worby *et al.*,

2006). Glycogen, however, has more branch points, which are arranged more uniformly than those in amylopectin (Zeeman *et al.*, 2010). Amylopectin has α-1,6 bonds occurring approximately every 24–30 residues and its structure is much more ordered. One model of amylopectin indicates that short chains (with a degree of polymerization (DP) of between 6-20) form clusters together and these are then linked together by longer chains (DP 20-70) (Waigh *et al*., 1996, 1997, 1998). The individual short chains within clusters form double helices (Fig. 1.1) that can crystalize, leading to the molecule's relative insolubility (Worby *et al.*, 2006). The helices are arranged together in ordered arrays that form crystalline lamellae, known as the semi-crystalline zones (Smith *et al.*, 1999). Amorphous lamellae (in which the amylopectin is far less organized (French, 1984; Jenkins *et al.*, 1993)) are found between the crystalline lamellae, resulting in a concentric pattern of alternating crystalline and amorphous lamellae (Smith *et al.*, 1999). Amylose is normally considered linear, although it does contain a small number of branch points (Ball and Morell, 2003) and the majority of this fraction can be found as single helices (Fig. 1.1), interspersed with amylopectin, within the amorphous zones (Jane *et al.*, 1992).

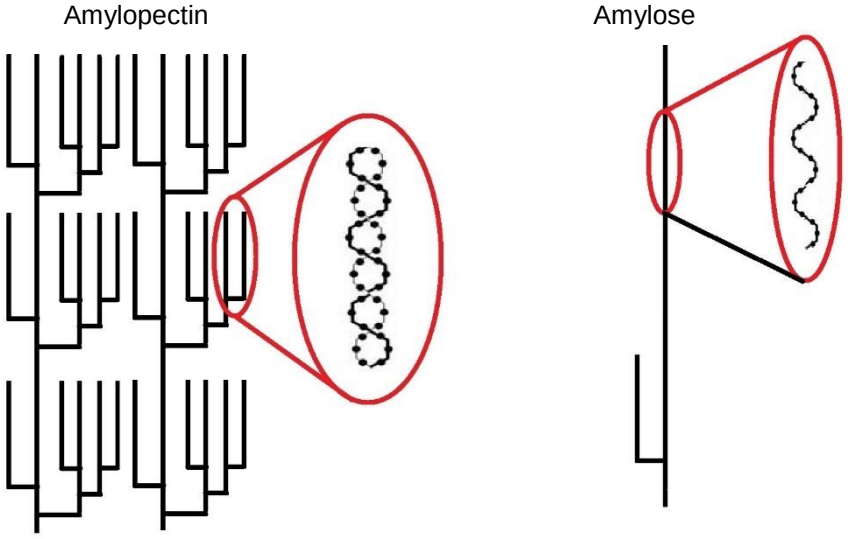

Figure 1.1 Starch structure. A schematic representation of amylopectin and amylose, and the structure of their constituent chains. Adjacent amylopectin chains form double helices, whilst amylose chains form single helices (figure adapted from Zeeman *et al.*, 2010).

Starch is one of the most important polysaccharides produced by plants and is the primary source of dietary carbohydrates. Proper turnover of the polymer is required for plant growth, as even small alterations in leaf starch turnover affect growth and metabolism (Stitt and Zeeman, 2012). Indeed, starch has been manipulated in leaves to improve plant growth (Zeeman *et al.*, 2010). Additionally, many industries make use of it and this will be discussed in more detail below. It can be categorized into two general functional types, transitory starch (which is found in photosynthetic tissue and which is formed during the day and degraded at night) and storage starch (which is found in storage organs such as seed and tubers and, which, is stored for months or years before degradation).

1.2.1 Industrial uses of starch

Starch is one of the most commonly produced polymers in nature (Kötting *et al.*, 2005), and is used in many industrial applications. For example, as a thickener in the food industry; in detergents, adhesives, and resins; as well as for the production of paper, processing of textiles, and purification of water (Kraak, 1993). Its structure affects characteristics that are influential in its application, including gelling and pasting strength, viscosity and stickiness (Lorberth *et al.*, 1998; Carciofi *et al.*, 2011). An example of this is starch phosphorylation, which has been shown to improve industrially relevant characteristics, which will be discussed in depth in section 1.2.3. Starch used in industry is mainly derived from plants that store it in high quantities in seeds, such as maize, although specific industries use potato starch due to its high phosphate content (Zeeman *et al*., 2010), which enables it to swell considerably when heated in water.

1.2.2 Starch metabolism

Both the biosynthesis and subsequent degradation of starch are closely regulated to allow the plant to adapt to changes in the environment through altering metabolism. The formation of starch requires the synthesis of α-1,4 glucans by starch synthase (SS) isoforms that use the activated sugar ADP-glucose, and add it to the non-reducing end of a glucose chain (Smith *et al.*, 1997). Introduction of α-1,6-glycosidic branches is catalyzed by starch branching enzymes (SBE) that cleave a section from an existing chain and transfer it to another chain (Smith *et al.*, 1999). Starch synthase and branching enzymes act simultaneously, with chains being lengthened whilst branch points are introduced (Nielsen *et al.*, 2002). There are

multiple, distinct isoforms of starch synthase and starch branching enzyme. Additionally, these isoforms are each encoded for by different genes (Smith *et al.*, 1999). Starch synthases in higher plants are encoded by six gene classes: GBSS (granule-bound starch synthase), SSI, SSII, SSIII, SSIV and SSV (Zeeman *et al.*, 2010; Nougué *et al.*, 2014). GBSS synthesizes amylose, and is bound closely to the starch granule (Zeeman *et al.*, 2010). The remaining SS isoforms synthesize the chains in amylopectin, with each isoform having distinct properties and roles (Zeeman *et al.*, 2010). Examination of amylopectin chain length distribution in mutant and transgenic plants lacking specific isoforms suggests that SSI preferentially elongates short chains, SSII elongates medium length chains, and SSIII elongates long chains (Tomlinson and Denyer, 2003). SSIV is thought to be involved in starch granule initiation (Roldán *et al.*, 2007; Szydlowski *et al.*, 2009), while the role of SSV has yet to be analyzed.

Starch branching enzymes can be categorized into two classes: class I and class II (Zeeman *et al.*, 2010) although these classes have also been referred to as classes B and A, respectively (Burton *et al.*, 1995). Downregulation of SBEI had little effect on starch structure and the relative amounts of amylose and amylopectin (Safford *et al.*, 1998). However, when SBEII was downregulated, there was an increase in amylose content (Jobling *et al.*, 1999). Plants lacking both isoforms contain substantially increased amylose due to the decreased ability to form the branches that are present within amylopectin (Schwall *et al.*, 2000).

Interestingly, it has become clear over the past twenty years that enzymes that synthesis the polymer are not the only ones required to determine its structure, with some degradative enzymes also being involved. Isoamylase type debranching enzymes catalyze the removal of branch points. Mutants lacking specific isoforms of this enzyme demonstrated changes in glucan accumulation and structure, accumulating a glycogen-like polysaccharide in addition to, or instead of, starch (Sumner and Somers, 1944; Matsuo *et al.*, 1987; Mouille *et al.*, 1996; Zeeman *et al.*, 2010). One model proposed by Ball *et al.* (1996) hypothesized that starch synthase and branching enzyme together synthesize ‘pre-amylopectin’, a highly branched and unorganized glucan, that is then acted on by debranching enzyme, allowing the formation of structured amylopectin. Lack of the debranching enzyme would mean that

the pre-amylopectin could not be degraded and would accumulate at the expense of starch.

1.2.3 Incorporation and removal of phosphate into starch

Starch phosphorylation is found in varying degrees in almost all plant species (Blennow *et al.*, 1998; Blennow *et al*., 2000; Carciofi *et al.*, 2011). The amount reported in vegetative storage organs (such as potato tubers) is greater than most other organs (Fernbach, 1904), containing around 8–33nmol/mg starch (Baunsgaard *et al.*, 2005), or approximately one phosphorylated glucose monomer for every 200–300 non-phosphorylated moieties (Hizukuri *et al.*, 1970). Starch from cereal endosperm contains very low concentrations of covalently bound phosphate, approximately 1nmol/mg (Carciofi *et al.*, 2011), although some phospholipids are often found associated with cereal endosperm starch granules (Kasemsumwan and Jane, 1996). About two thirds of the phosphate monoesters are linked by covalent bonds to the C-6 position, whilst approximately one third is linked to the C-3 position (Hizukuri *et al.*, 1970). In some cases, it has been proposed that up to 1% of the phosphate could be linked to the C-2 position (Hizukuri *et al.*, 1970; Takeda and Hizukuri, 1982).

Starch is phosphorylated within plastids and its presence is linked with other aspects of starch metabolism (Carciofi *et al.*, 2011), as plants lacking starch bound phosphate are unable to degrade leaf starch (Yu *et al.*, 2001; Ritte *et al.,* 2002; Nashilevitz *et al.*, 2009; Vriet *et al.*, 2010; Hirose *et al.*, 2013; Ma *et al.*, 2014). Phosphorylation is catalyzed initially by glucan, water dikinase (GWD) and, downstream, by phosphoglucan, water dikinase (PWD) (Ritte *et al.*, 2002; Kötting *et al.*, 2005) enzymes. Both of these polypeptides transfer the β-phosphate of ATP to a glucose residue (Ritte *et al.*, 2002), but GWD phosphorylates the C6 position, and PWD the C-3 position within chains that have already been phosphorylated by GWD (Kötting *et al.*, 2005). Phosphorylation is the only known naturally-occurring covalent modification of starch and contributes to several of its functional properties, including its low pasting temperature, high viscosity, clarity, stickiness, gel strength, and freeze-thaw stability (Lorberth *et al.*, 1998; Carciofi *et al.*, 2011; Santelia and Zeeman, 2011).

Phosphorylation is also important for the promotion of starch degradation (Edner *et al.*, 2007), as it hydrates and solubilizes the surface of the starch granule allowing access to amylolytic enzymes (Ritte *et al.*, 2002; Blennow and Engelsen,

2010). Decreased phosphorylation reduces starch degradation in mutant plants, leading to enhanced starch accumulation and a starch excess phenotype in the leaves (Lorberth *et al.*, 1998; Yu *et al.*, 2001). The severity of this starch excess phenotype shows an inverse correlation to the degree of phosphorylation (Yu *et al.*, 2001). This has been demonstrated in transgenic potato and mutant *Arabidopsis*, rice, lotus and tomato plants, where GWD deficiency caused decreased phosphorylation and a resultant decreased in ability to degrade leaf starch (Ritte *et al.,* 2002; Nashilevitz *et al.*, 2009; Vriet *et al.*, 2010; Hirose *et al.*, 2013; Ma *et al.*, 2014). The development of a starch excess phenotype of transgenic plants led to a 50-90% increase in dry matter, which would be beneficial in crops used for feed (Lorberth *et al.*, 1998), for example in crops such as grasses, lucerne (Humphries, 2012), sugar beets (Dohm *et al.*, 2014) and Japanese radish (Van Zyl and Dannhauser, 2009; Mutegi *et al.*, 2013. GWD has also been shown to affect starch degradation in non-photosynthetic tissue, as lowered levels of GWD also reduce cold-induced sweetening in potato tubers, a phenomenon that occurs when starch is converted into soluble sugars during prolonged cold storage of potatoes (Lorberth *et al.*, 1998).

Phosphorylation of the surface of starch during breakdown in turn increases PWD activity, further increasing phosphorylation, as PWD exhibits a higher affinity for phosphorylated starch (Kötting *et al.*, 2005). This has potential applications in industry, as hyper-phosphorylated starch could result in biopolymers that are more readily degraded (Carciofi *et al.*, 2011). Alterations in the phosphorylation of starch by manipulating enzymes involved in its metabolism are, therefore, of commercial importance.

Although phosphorylation occurs during starch breakdown, removal of the phosphate by phosphatase enzymes is also required for degradation (Kötting *et al.*, 2009) due to the inability of β-amylase (a glucan hydrolytic enzyme) to degrade past a phosphate group (Takeda and Hizukuri, 1981; Fulton *et al.*, 2008). Two starch phosphatases, Starch Excess4 (SEX4) and Like Sex Four2 (LSF2) have been identified which accomplish this and mutations in either reduce the ability of plants to degrade leaf starch (Kötting *et al.*, 2009; Santelia *et al.*, 2011).

1.3 Glycogen

Glycogen is a polymer that is found in most bacteria, archaebacteria, fungi, and animals (Gentry *et al.*, 2007). It is stored in the form of glycogen bodies, is the only intracellular storage polysaccharide found in archaea and bacteria (Rehm, 2010). It is highly branched (Fig. 1.2) and is composed of glucose residues linked by α-1,4-glycosidic linkages, with α-1,6 branchpoints occurring every 8–14 residues (Gentry *et al.*, 2007; Worby *et al.*, 2006). Up to 10% of all the glycosidic bonds within glycogen are α-1,6, and its specific branching structure causes it to be water soluble (Manners, 1991). Most bacteria synthesize glycogen from ADP-glucose whilst mammals, fungi, and some bacteria utilize UDP-glucose (Roach *et al.*, 1998). Ball and Morell (2003) report that the unit particles of glycogen are small, with diameters of less than 50 nm. The pathway of glycogen synthesis is similar in all organisms that accumulate it.

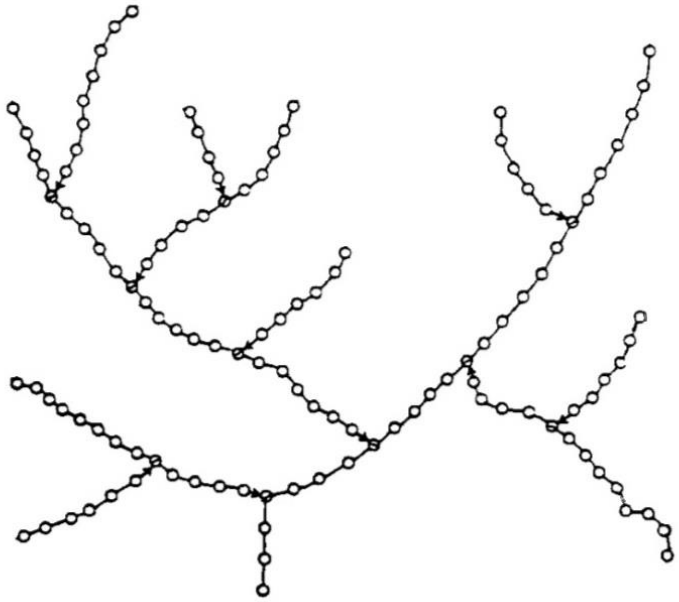

Figure 1.2 Glycogen structure. A schematic representation of the α-1,4-linked chains with α-1,6-branch points (figure adapted from Manners, 1991).

1.3.1 *E. coli* glycogen metabolism

In *E. coli* the genes encoding the enzymes affecting both the synthesis and structure of glycogen are found in two neighboring tandemly expressed operons (Preiss and Romeo, 1989). Many bacteria, including *E. coli*, contain a ADP-glucose pyrophosphorylase (encoded by the *GlgC* gene) that catalyzes the synthesis of ADP-

glucose, as the first committed step in the synthesis of glycogen (Ball and Morell, 2003). Synthesis of the polymer requires two enzymes: glycogen synthase (Encoded by *GlgA*) to form α-1,4-glycosidic linkages and the branching enzyme (Encoded by *GlgB*) to form α-1,6-glycosidic branchpoints (Tagliabracci *et al.*, 2007). Although glycogen synthase catalyzes the addition of moieties to existing glucans, in most bacteria it cannot prime this initial reaction (Ball and Morell, 2003). An exception to this within bacteria is *Agrobacterium*, where the enzyme is capable of priming this reaction. Branching enzyme then cleaves existing α-1,4-glucans and transfers segments of these to α-1,6. positions on a polyglucan, hereby introducing α-1,6-linked branch points to the existing α-1,4-linked chains (Ball and Morell, 2003). This is clearly very similar to the starch biosynthetic pathway (see section 1.2.2) and the enzymes involved show similarity on the primary protein level to the ADP-glucose pyrophosphorylase, starch synthase and starch branching enzymes that synthesize starch.

Catabolism of glycogen requires the activity of several enzymes. A glycogen phosphorylase (GlgP) removes glucose moieties through phosphorolysis (Alonso-Casajús *et al.*, 2006), a debranching enzyme degrades the α-1,6 branchpoints (Dauvilée *et al.*, 2005), while a maltodextrin phosphorylase (MalP) removes the linear malto-oligosaccharides that are the result of these actions (Strydom *et al.*, 2017).

1.3.2 Incorporation of phosphate into glycogen

1.3.2.1 Engineering *E. coli* with increased glycogen phosphate

The pathway of starch phosphate incorporation was discussed in section 1.2.3 and it is clear that it is incorporated by two enzymes, GWD and PWD. Experiments have demonstrated that GWD can also catalyze the phosphorylation of glycogen when expressed in bacteria (Lorberth *et al.* 1998, Viksø-Nielsen *et al.* 2002). This higher phosphate content in *E. coli* was associated with an increased accumulation of glycogen, resulting in a glycogen excess phenotype. Cells expressing GWD store up to 50% more glycogen, with more than one glycogen body being found in each cell (Viksø-Nielsen *et al.*, 2002). Potato GWD therefore increased phosphorylation in polyglucans in both plants and *E. coli*, however, this phosphorylation resulted in different phenotypic responses in each case. Phosphorylation of glycogen also results in an altered structure whereby the polymer exhibits different chain-length distribution. Glycogen purified from cells expressing GWD had a higher proportion of short chains

(DP 3–10), compared with the control (DP 12) (Viksø-Nielsen *et al.*, 2002). This most likely indicates that the glycogen polymerizing enzymes exhibit different activities on phosphorylated substrate leading to changes in structure.

1.3.2.2 Phosphorylation of mammalian glycogen

Over the past decade, evidence has been presented that mammalian glycogen contains some covalently bound phosphate (See Roach *et al.*, 2012 and Sullivan *et al.*, 2017 for recent reviews). Indeed, the phosphorylation of glycogen in humans is linked to a serious genetic disease, Lafora disease, a fatal progressive myoclonic epilepsy (Gentry *et al.*, 2007; Tagliabracci *et al.*, 2007). Individuals who suffer from this disease accumulate insoluble polyglucosans (Lafora bodies or LBs) within cytoplasm (Harriman *et al.*, 1955; Schwarz and Yanoff, 1965; Carpenter and Karpati, 1981), particularly in cells of organs that do not typically store large amounts of glycogen, such as the heart, skin, and neurons (Gruetter, 2003). They also develop them in organs that accumulate large amounts of glycogen such as liver and muscles (Gruetter, 2003). LBs have the same backbone as glycogen (α-1,4-glycodisic linkages), but contain fewer branches (α-1,6-linkages), resulting in an insoluble crystalline structure that is the causal reason for their build-up (Worby *et al.*, 2006). Despite most studies referring to LBs as insoluble glycogen, they are structurally more similar to amylopectin than glycogen (Gentry *et al.*, 2007). LBs are also considerably more phosphorylated than normal mammalian glycogen, which is believed to contribute both to their accumulation and their lack of degradation (Tagliabracci *et al.*, 2011). Glycogen from mouse models of Lafora disease, where the Laforin gene is deleted, also shows elevated phosphorylation (Tagliabracci *et al.*, 2007).

Mutations in the *Laforin* gene, which leads to the development of this disease, affect a phosphatase that binds glycogen and removes phosphate from it (Tagliabracci *et al.*, 2007). This phosphatase is both structurally and functionally similar to the starch phosphatases SEX4 and LSF2 (Gentry *et al.*, 2007, Meekins *et al*., 2015) It is assumed that its lack leads to glycogen phosphate build up as there is no other protein able to remove it. Recently it has been shown, however, that the removal of phosphate itself is unimportant for development of the disease as an inactive Laforin protein was able to complement mice mutated in *Lafora* (Gayarre *et al.*, 2014). It is now thought that Laforin has two roles, firstly it dephosphorylates glycogen and secondly helps

determine chain length within glycogen, and it is this second role that is important for the development of Lafora disease (Nitschke *et al.*, 2017).

Although the pathway of glycogen de-phosphorylation appears to have been elucidated, the manner by which phosphate becomes incorporated is less well understood. One theory proposes that glycogen synthases incorporate the phosphate (Tagliabracci *et al.*, 2011) and it has been demonstrated that the protein can do this *in vitro* using the β-phosphate of UDP-glucose as a phosphate donor (Contreras *et al.*, 2016), but its role has not been demonstrated *in vivo*.

1.3.2.3 Phosphorylation of bacterial glycogen

Given the presence of phosphate in both starch and mammalian glycogen the question arises if it is present in storage polyglucans form other organisms. There are a small number of studies where phosphate in bacterial glycogen has been found. In studies examining the role of the GWD protein in phosphorylating polyglucans, both Lorberth *et al.* (1998) and Viksø-Nielsen *et al*. (2002) demonstrated that glycogen from wild type *E. coli* contains a small amount of phosphate bound as monoesters at the C-6 position. This indicates that the enzymes and biochemical components required for phosphorylation must also be present in bacteria.

A study (Nepembe, 2009) performed at the Institute for Plant Biotechnology (IPB) demonstrated that expression of the plant SEX4 protein in *E. coli* led to cells that did not accumulate glycogen. As SEX4 acts as a polyglucan phosphatase it was assumed that it removed phosphate from glycogen and proposed that this led to less glycogen being synthesized. In that study it was hypothesized that two polyglucan phosphorylases, GlgP and MalP, may be able to phosphorylate *E. coli* glycogen. These two reversible enzymes can either remove or incorporate glucose into polyglucans. In the synthesis direction, they use G1P as substrate and release inorganic phosphate, while in the degradation direction they form G1P by removing a glucose residue by phosphorolysis (Park *et al.*, 2011). It was proposed that phosphate could be incorporated into a polyglucan as glucose-6-phosphate by these enzymes using glucose-1,6-bisphosphate (GBP) as a substrate (Nepembe, 2009). This theory however, remains untested and the aim of this study is to examine this theory. The remainder of this literature review will, therefore, examine MalP and GlgP in more detail.

1.4 The GlgP and MalP enzymes of *E. coli*

1.4.1 Functions of GlgP and MalP in metabolism

MalP and GlgP were, for many years, thought to be involved in separate metabolic pathways in *E. coli*. GlgP was assumed to be involved only in glycogen metabolism, while MalP was thought to affect maltodextrins (linear α-1,4 linked polyglucans synthesized from maltose). Both the Glg and Mal operons are separate in *E. coli*, although they are located only about 12 kilo base pairs apart within the genome. Both glycogen and maltodextrins are similar in structure, so it is perhaps unsurprising that more recent evidence indicates that the enzymes involved in metabolism of these two polyglucans can be involved in both pathways, as this would introduce redundancy into polyglucan metabolism. When *E. coli* cells are grown on maltose for example, they produce glycogen through extending maltose to produce linear α-1,4 linked malto-oligosaccharides (MOS) that can be acted upon by the branching enzyme GlgB (Park *et al.*, 2011).

MalP was originally thought to be involved only in degrading MOS that are produced when *E. coli* are grown on maltose, however, both MalP and GlgP have been demonstrated to be involved in glycogen metabolism (Alonso-Casajús *et al.*, 2006, Strydom *et al.*, 2017). Mutations in GlgP lead to *E. coli* accumulating increased amounts of glycogen, the constituent chains of which are lengthened. It was proposed that GlgP acts to degrade external glycogen chains and so when it is absent, these chains become longer (Alonso-Casajús *et al.*, 2006). A recent study has also demonstrated a role for MalP in glycogen degradation, but only when both GlgP and the debranching enzyme GlgX are also absent. A triple mutant lacking MalP, GlgP and GlgX accumulates more glycogen when grown on solid media than the *Δglgp/Δglgx* double mutant (Strydom *et al.*, 2017) and it was proposed that MalP removes linear chains released by GlgX.

1.4.2 GlgP

1.4.2.1 Kinetics of GlgP

The K_m values of *E. coli* GlgP for synthesis and phosphorolysis for various substrates have been calculated (Boeck and Schinzel, 1996; Chen and Segel, 1968b), and are presented in Table 1.2. Inhibitors of GlgP include ADP-glucose, Ag^{+}, Cu^{2+},

Hg^{2+}, and Zn^{2+} (Chen and Segel, 1968b). It is activated by Na_2SO_4 and 5'-AMP (Chen and Segel, 1968b). The pH optimum for GlgP is pH 6.7–6.9 (Chen and Segel, 1968b).

Table 1.2 GlgP K_m values. Values were determined by Boeck and Schinzel (1996) and Chen and Segel (1968b).

Substrate	**K_m value (mM)**
Synthesis	
glucose-1-phosphate	1
maltotetraose	3.6
maltotriose	24.5
Phosphorolysis	
maltoheptaose	0.2
maltotetraose	3.9
phosphate	0.5

1.4.2.2 Regulation of GlgP

As is the case with most enzymes involved in glycogen metabolism, GlgP is subject to regulatory systems. The expression of the *glg* genes is complex, and involves the global carbon storage regulator CsrA (Yang *et al.*, 1996; Baker *et al.*, 2002); the cyclic AMP/catabolite gene activator protein system (Romeo and Preiss, 1989); and the stringent response (Romeo *et al.*, 1990). Moreover, GlgP is affected by the sugar phosphotransferase transport system (Seok *et al.*, 1997, 2001). The protein is activated through interactions with the un-phosphorylated form of HPr, a constituent of the phosphotransferase system. Intracellular Mg^{2+} levels are also linked to the system through the PhoP-PhoQ regulatory system (Montero *et al.*, 2009).

1.4.3 MalP

1.4.3.1 Kinetics of MalP

There is less information available for the kinetics of *E. coli* MalP, although Becker *et al.* (1994) determined the K_m of MalP for a variety of substrates (Table 1.3). Inhibitors and activators for *E. coli* MalP have yet to be determined. The pH optimum for MalP is pH 6 (Zhang *et al.*, 2008).

Table 1.3 MalP K_m values. Values were determined by Becker *et al.* (1994).

Substrate	**K_m value (mM)**
Synthesis	
glucose-1-phosphate	1
2-Deoxy-Glc-1-P	3.3
Phosphorolysis	
glucose	0.5
2-Deoxy-Glc-Glc	>15

1.4.3.2 Regulation of MalP

The maltose/maltodextrin system is comprised of five operons with 10 genes in total encoding proteins that function in the uptake of maltose and maltodextrins (Park *et al.*, 2011). These genes encode an ABC transporter, three cytoplasmic enzymes (MalQ, MalP, and MalZ), and one periplasmic enzyme (MalS) (Boos and Shuman, 1998). Glucose is removed from the reducing end by MalQ (amylomaltase), which is the primary enzyme in the utilization of maltose (Monod and Torriani, 1950; Pugsley and Dubreuil, 1988; Wiesmeyer and Cohn, 1960). It is a 4-α-glucanotransferase that preferentially acts on maltose and shorter maltodextrins, allowing the remaining residue to be transferred to other maltodextrins to form longer chains of maltodextrins (Palmer *et al.*, 1973). Maltodextrin phosphorylase (MalP) recognizes maltotetraose and longer maltodextrins and causes phosphorolysis of glucose from the nonreducing ends of maltodextrins, yielding G1P (Park *et al.*, 2011). These two intermediate products from MalQ and MalP (glucose and G1P) enter glycolysis, thus allowing *E. coli* to efficiently utilize maltose and maltodextrins (Park *et al.*, 2011). MalP allows for the formation of glycogen, even without the presence of glycogen synthase (Park *et al.*, 2011).

1.5 Conclusions and further prospects

Although glycogen does not have industrial applications as a biopolymer, this study is still of scientific, industrial, and medical relevance as it will help to develop a deeper understanding of how bacteria store and chemically alter glycogen. The importance of studying this system in *E. coli* is apparent, as it is a model organism to study phosphorylation of polyglucans (Viksø-Nielsen *et al.*, 2002). Starch and

glycogen have very similar biosynthetic pathways, including substrates and enzymes and presence of enzymes required for phosphorylation (Viksø-Nielsen *et al.*, 2002). Furthermore, recombinant expression of the GWD known to phosphorylate starch also increased phosphate incorporation into glycogen in *E. coli*, suggesting compatibility of the two pathways (Viksø-Nielsen *et al.*, 2002). The dynamic balance of synergistic processes affecting glycogen and starch metabolism and phosphorylation is an important one to understand and manipulate in order to optimize polyglucan metabolism for commercial use (Kötting *et al.*, 2009). If starch structure can be altered by manipulating the activities of the GlgP and MalP enzymes, this would alter its physicochemical properties and potentially allow it to be used in novel technical processes.

1.6 Aims and objectives

The long-term goal of this project within the context of other work in this field is to improve the understanding of the biochemistry and metabolism of glycogen and starch. This knowledge could then be applied to alter various properties of these polymers to enhance their usefulness in industrial applications. More specifically, the overall aim of this project is to examine the potential biochemical mechanisms of incorporation of phosphate into glycogen in *E. coli* by examining the functioning of two phosphorylase enzymes, to determine whether GlgP and MalP incorporate phosphate into polyglucans using glucose-1,6-bisphosphate as a substrate. This will be examined in both *in vitro* and *in vivo* systems.

Objectives for this project include:

- Production and purification of recombinant GlgP and MalP
- Biochemical analysis of the purified proteins to examine their potential to phosphorylate polyglucans
- Extraction and purification of glycogen from different strains of *E. coli* mutants
- Analysis of glycogen from mutant strain

Chapter 2: GlgP and MalP can incorporate phosphate into polyglucans *in vitro*, but not *in vivo*.

2.1 Introduction

Over the past decade it has become clear that phosphate is present in glycogen found in both mammals and bacteria, as well as the similarly structured plant storage product, starch (See chapter 1 section 1.3.2 and the reference present within). Starch is known to be phosphorylated by two dikinases, the glucan and phosphoglucan, water dikinases (GWD and PWD respectively; Ritte *et al.* 2006). Mammalian glycogen has been hypothesized to be phosphorylated by glycogen synthases (Tagliabracci *et al.*, 2011), with the enzyme being capable of doing so *in vitro* using the β-phosphate of UDP-glucose as a phosphate donor (Contreras *et al.*, 2016); however, there is no data that confirms this *in vivo*.

Little is known about phosphorylation of bacterial glycogen. Two studies have reported the presence of phosphate at the 6-position of glucose moieties within *E. coli* glycogen (Lorberth *et al.* 1998; Viksø-Nielsen *et al*. 2002) while Nepembe (2009) observed that expression of a starch phosphatase in the same bacteria inhibited glycogen accumulation, which implies that glycogen phosphate plays a role in its metabolism. These observations indicate that *E. coli* glycogen, like starch and mammalian glycogen contains some covalently bound phosphate, but the mechanism by which it becomes incorporated is currently unknown. Elucidating this mechanism would be interesting on two levels. Firstly, it would allow an understanding of the role of glycogen bound phosphate in *E. coli* metabolism and, secondly, it could be used biotechnologically to increase phosphate in starch to make it more usable by industry (Zeeman *et al*., 2010).

Lorberth *et al.* (1998) and Nepembe (2009) suggested that phosphorylase enzymes could be involved in phosphorylation of polyglucans, using glucose-1,6-bisphosphate as a substrate. *Escherichia coli* contain two polyglucan phosphatases, the glycogen phosphorylase (GlgP) and the maltodextrin phosphorylase (MalP). Both

have been demonstrated to be involved in glycogen metabolism (Alonso-Casajús *et al*., 2006; Park *et al*., 2011; Strydom *et al.*, 2017). This study examines if they are involved in glycogen phosphorylation, firstly by testing whether or not they can accomplish this *in vitro* and, secondly by examining if starch bound phosphate is present in a *Δmalp/Δglgp* double mutant.

2.2 Methods and materials

2.2.1 Chemicals

All chemicals and reagents used were of analytical grade. Unless otherwise stated, chemicals were obtained from Sigma (St. Louis, USA) or Roche (Mannheim, Germany). Enzymes were acquired from Sigma (St. Louis, USA) or Megazyme (Bray, Ireland). Protein purification columns were obtained from Macherey-Nagel (Düren, Germany). Paramagnetic particles for protein purification were obtained from Promega (Madison, USA), and the thrombin cleavage kit was obtained from Sigma-Aldrich (St. Louis, USA). Solutions were prepared with deionized distilled water (ddH_2O, Millipore).

2.2.2 Bacterial genotypes and plasmids

Bacterial genotypes used in this study are shown in Table 2.1, while plasmids are described in Table 2.2.

Table 2.1 Genotypes of bacterial strains

Name	Source	Description
BW25113	Datsenko and Wanner, 2000	*Δ(araD araB)567 ΔlacZ4787*(::rrnB-3) *λ- rph-1 Δ(rhaD-rhaB)568*
JW3391 (ΔGlgP(kan))	Baba *et al.*, 2006	BW25113 *ΔglgP-761::kan*
JW5689 (ΔMalP(kan))	Baba *et al.*, 2006	BW25113 *ΔmalP-751::kan*
ΔGlgP	Strydom *et al.*, 2017	BW25113 *ΔglgP-761*
ΔmalP	Strydom *et al.*, 2017	BW25113 *ΔmalP-751*
ΔGlgP/MalP(kan)	Strydom *et al.*, 2017	BW25113 *ΔglgP-761 ΔmalP-751::kan*

ΔMalP/GlgP(kan)	Strydom *et al.*, 2017	BW25113 *ΔmalP-751 glgP-761::kan*
E. coli BL21-AI	Life Technologies, Invitrogen	F^- *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm araB::T7RNAP-tetA*
E. coli BL21 Rosetta-gami	Novagen, Merck Biosciences	P15A, Cam^R, Kan^R, Tet^R, *Δ trxB/gor*

Table 2.2 Plasmids used in the study

Name	Source	Description
pACAG	Kossmann *et al.* 1999	Cam^R, Tet^R, IPTG inducible *glgC16* expression
pDEST17.MalP	Jewell, 2015	Amp^R, His-tag, IPTG inducible vector
pET-41a.GlgP	This study	Kan^R, GST-tag, IPTG inducible vector

2.2.3 Production of GlgP protein expression vector

The *glgP* gene (forward primer: 5‟CACCATGAATGCTCCGTTTACATA3‟; reverse primer: 5‟ACTTACAATCTCACCGGATCGA3‟) was amplified by PCR from *E. coli* genomic DNA. The amplicon was extracted and purified, then visualized on a 1% (w/v) agarose gel. The gene was recombined into pET-41a, producing pET-41a.GlgP. The presence of the *glgP* gene was confirmed by PCR.

2.2.4 Bacterial transformation

Plasmids were transformed into heat shock competent *E. coli* cells and selected on solid media containing appropriate antibiotics.

2.2.5 Production of recombinant protein

Single colonies of *E. coli* cells containing a vector allowing expression of a gene expressing MalP or GlgP, fused in frame with a His-Tag or GST-Tag, respectively, were used to inoculate starter cultures of 2 mL lysogeny broth (LB)

media (1% (w/v) peptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl) with appropriate antibiotics, which was cultured overnight at 37 °C with agitation. The 2 mL starter cultures were used to inoculate 200 mL of the same medium. When the OD_{600} reached approximately 0.4, L-arabinose was added to a final concentration of 0.2% (w/v) for induction of MalP, and isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM for the induction of GlgP. The resulting cells were grown at 28 °C overnight with shaking. The culture was centrifuged at 10000 × g at 4°C for 15 minutes to pellet cells before the supernatant was discarded. Cells were re-suspended in 10 mL pre-chilled buffer and sonicated ten times on ice for 15 second bursts, with 15 seconds non-sonication in between. The lysate was centrifuged for 30 minutes at 10000 × g at 4 °C and supernatant filtered through a 0.45 µm cellulose acetate membrane. The buffer used for MalP extraction was NPI-10 buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10mM imidazole (pH 8.0)), whilst GlgP was extracted in protein extraction buffer (50 mM Tris-HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT)).

2.2.6 Purification of recombinant protein

MalP purification was performed by immobilized metal ion affinity chromatography (IMAC) using 1 mL Nickel-nitrilotriacetic (Ni-NTA) agarose-linked columns. The supernatant was passed through the column and washed with 20 column volumes of NPI-20 buffer (50 mM NaH_2PO_4, 300 mM NaCl and 20mM imidazole (pH 8.0)). Histidine-tagged (His-tagged) protein was eluted in 1 mL fractions with NPI-250 buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250mM imidazole (pH 8.0)), with a gradient from 0-100% elution buffer over 10 column volumes, and 100% elution buffer for another 65 column volumes. Fractions were tested for activity and the most active of these were combined, dialyzed against 50 mM Tris-HCl (pH 7.0), and stored at -20 °C.

GlgP purification was performed using the MagneGST protein purification system, whereby the GST-fusion GlgP protein was isolated from crude lysate by use of paramagnetic particles as described by the manufacturer (Promega). The GST tag was cleaved from eluted protein using the Thrombin CleanCleave kit. The sample, containing both GST and MalP polypeptides was dialyzed against 50 mM Tris-HCl (pH 7.0) before the MagneGST kit was used to remove the GST tag. Fractions were tested for activity and the most active of these were combined and stored.

2.2.7 Phosphorylase activity assays

The activity of MalP and GlgP samples were tested spectrophotometrically using an enzyme-coupled assay examining liberation of glucose-1-phosphate. Increases in OD_{340} was measured after addition of enzyme to 200 µL assay buffer (50 mM potassium phosphate buffer (pH 7.0), 2 mM NAD^+, 20 µM glucose-1,6-bisphosphate (GBP), 5.6 U/mL glucose-6-phosphate dehydrogenase (G6PDH, from *L. mesenteroides*), 1 U/mL phosphoglucomutase (PGM, from rabbit muscle), and 1% (w/v) substrate. Dextri-maltose was used as a substrate for MalP activity assays, whilst glycogen (from oyster) was used for GlgP assays.

2.2.8 SDS-polyacrylamide gel electrophoresis

Protein fractions were separated by discontinuous 10% (w/v) denaturing polyacrylamide gel electrophoresis (PAGE) with sodium dodecyl sulfate (SDS) at 120 V. A pre-stained protein ladder (PageRuler Plus, Thermo Fisher Scientific, Waltham, USA) was used as a molecular weight marker. Upon completion, the gels were stained overnight with Coomassie Brilliant Blue R-250 stain (45% (v/v) methanol and 10% (v/v) acetic acid), and de-stained using an aqueous solution containing 45% (v/v) methanol and 10% (v/v) acetic acid.

2.2.9 In gel detection of phosphorylase activities

Protein samples were separated by non-denaturing PAGE. The resolving gel contained 10% (w/v) polyacrylamide and 1% (w/v) glycogen (from oyster). Separation took place at 4 °C at 85 V. The gels were incubated overnight in 3 M acetic acid (pH 6.0) with 10 mM glucose-1-phosphate with agitation at room temperature (Nepembe, 2009). Once the incubation buffer was removed, the gels were stained with Lugol's solution (4% (w/v) KI, 2% (w/v) I_2) for five minutes, and de-stained with ddH_2O.

2.2.10 Enzyme quantification and storage

Protein concentrations for the purified enzymes were determined by the Bradford method (Bradford, 1976), using a protein assay dye (Bio-Rad, Munich), with bovine serum albumin (BSA) as a standard. Known quantities of purified MalP and GlgP were stored at -20 °C with 10% (v/v) glycerol.

2.2.11 *In vitro* incubations

Incubations containing 0.75 µg purified protein in 150 µL incubation buffer (50 mM Tris-HCl (pH 6.8), 0.1% (w/v) sodium azide (NaN3), 0.3 mg glycogen (from oyster), and various concentrations of either glucose-1-phosphate (G1P), GBP or combinations of GBP and G1P) were conducted over two hours at 37 °C with agitation at 500 rpm. Separate incubations for active protein, or heat inactivated protein (30 minutes at 100 °C) were set up. Polymer was precipitated by the addition of molecular grade ethanol to a final concentration of 90% (v/v), followed by storage at -20 °C for one hour, and centrifugation for 10 minutes at 10000 × g at 4 °C before the supernatant was discarded.

2.2.12 Digestion of polyglucan to glucose and glucose-6-phosphate

Polymers were digested according to Nielsen *et al.* (1994). Acid hydrolysis was performed on the precipitated glycogen by re-suspending it in 400 µL 0.7 M HCl and heating it to 95 °C with agitation for three hours, after which it was neutralized by the addition of 400 µL 0.7 M KOH.

2.2.13 Enzymatic determination of glucose and glucose-6-phosphate content

Glucose and glucose-6-phosphate contents were determined spectrophotometrically. Glucose was determined by the addition of 10 µL sample to 200 µL assay buffer (300 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM NAD^+, and 1 mM ATP). After the sample had reached stable OD_{340}, hexokinase (0.50 U) and glucose-6-phosphate-dehydrogenase (0.25 U) were added to begin the reaction. The change in OD_{340} was recorded and used to calculate the quantity of glucose present in each sample. Glucose-6-phosphate was measured by adding 100 µL sample to 200 µL assay buffer (300 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, and 1 mM NAD^+). After addition of G6PDH (0.5 U) the change in OD_{340} was recorded and used to calculate G6P quantities.

The change in optical density in the controls (boiled protein) was subtracted from that of the active protein to give the quantity of polymer incorporated by the enzyme.

2.2.14 Measurement of inorganic phosphate

Phosphate was measured using a commercially available kit according to the manufacturer's instructions (EnzChek Phosphate Assay Kit, Molecular Probes, Eugene, USA).

2.2.15 ^{31}P nuclear magnetic resonance

Standards of G1P, G6P, GBP, and inorganic phosphate (P_i) (all with NAD^+ as an internal standard) were prepared in solution and analyzed with NMR. Precipitated polymer from incubations with MalP and 5 mM substrate was re-suspended in 800 µL ddH_2O, to which D_2O (10% (v/v)) and NAD^+ (1 mg/mL) were added before being filtered to rid the solution of any solid particles. NMR was performed at 300 MHz on a Varian VNMRS 300 Liquid State NMR Spectrometer (Agilent Technologies, Santa Clara, USA).

2.2.16 *E. coli* glycogen extraction

E. coli cultures were grown in 2mL liquid Kornberg Media (KB, 1.1% (w/v) K_2HPO_4, 0.85% (w/v) KH_2PO_4, and 0.6% (w/v) yeast extract) with 34 µg/mL chloramphenicol and 15 µg/mL tetracycline overnight at 37 °C with agitation. 150 µL of each of the cultures was transferred to 5 mL KB media (1% (w/v) glucose, 34 µg/mL chloramphenicol, and 15 µg/mL tetracycline), cultured for 6 hours, and then plated onto solid KB (1% (w/v) bacterial agar, 1% (w/v) glucose, 34 µg/mL chloramphenicol, and 15 µg/mL tetracycline). The plated cultures were grown at 37 °C overnight. The cells were scraped off the plates and stored at -20 °C.
Glycogen was extracted and isolated according to Park *et al.* (2011). The cells were thawed and re-suspended in 50 mM sodium acetate (pH 4.5), vortexed thoroughly, boiled for 10 minutes with agitation, and sonicated three times for five minutes (output power 6 watts, 60% maximum duty, at room temperature). Centrifugation for 20 minutes at 10000 × g at 25 °C followed to pellet the cells. The supernatant was transferred to a new centrifuge tube and two volumes molecular grade ethanol were added to precipitate the glycogen, which was collected by centrifugation for 20 minutes at 10000 × g at 4 °C. The pelleted glycogen was air-dried overnight.

2.2.17 Data analyses

Data analyses were performed using Excel (Version 1708, Microsoft, Redmond, USA) aided by Daniel's XL Toolbox add-in for Excel (version 6.60, Daniel

Kraus, Würzburg, Germany). NMR data were analyzed using MestReNova (version 10.0.2-15465, Mestrelab Research, Santiago de Compostela, Spain).

2.3 Results

2.3.1 Purification of recombinant protein

GlgP and MalP were purified by use of paramagnetic particles and affinity chromatography respectively. Samples were analyzed by SDS polyacrylamide gel electrophoresis to examine whether the protein had been isolated effectively (Fig. 2.1 A), and by non-denaturing PAGE to confirm activity of the purified protein (Fig. 2.1 B).

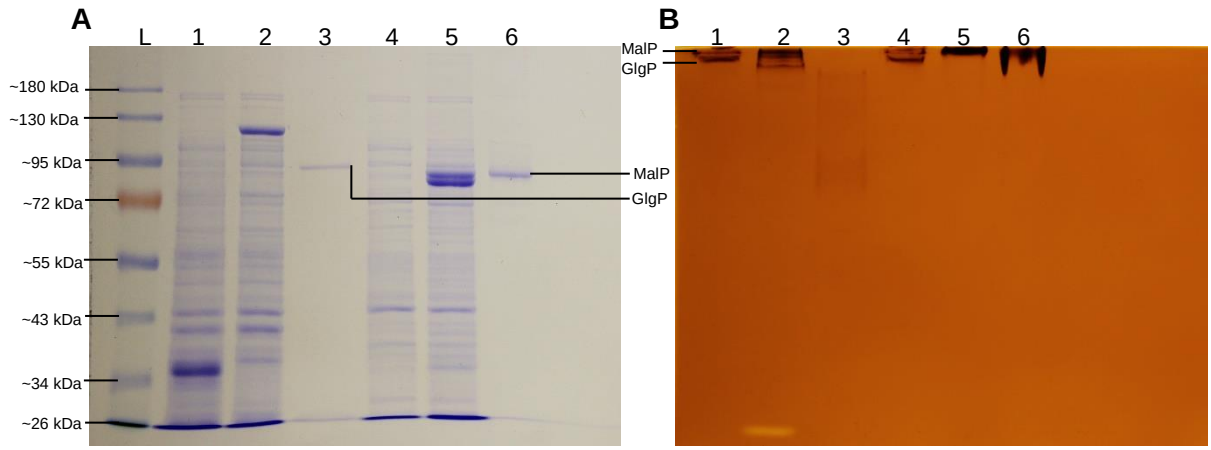

Figure 2.1 Purification of recombinant protein. A, 10% (w/v) SDS-PAGE gel showing a protein ladder (L) and protein purification. B, 10% (w/v) non-denaturing PAGE gel with 1% (w/v) glycogen to show enzyme activity. A and B, lane 1: empty pET-41a crude extract; lane 2: pET-41a.GlgP crude extract; lane 3: purified GlgP; lane 4: empty pRSET B crude extract; lane 5: pDEST17.MalP crude extract; lane 6: purified MalP.

GlgP (Fig. 2.1 A, lane 3) and MalP (Fig. 2.1 A, lane 6) were purified successfully from crude extracts (Fig. 2.1 A, lanes 2 and 5). There is one clear band for each purified protein, but some other minor ones are also present. The non-denaturing PAGE gel with shows MalP and GlgP activity. There is activity in the lanes of the empty vectors (Fig. 2.1 B, lanes 2 and 5), as well as the purified proteins (Fig. 2.1 B, lanes 3 and 6) The purified GlgP (Fig. 2.1 B, lane 3) shows activity further down in the gel than can be seen for GlgP activities in the lanes with the crude extracts.

2.3.2 Enzyme incubations

To examine whether GlgP and MalP were able to incorporate phosphate into the polymer it synthesizes, incubations were performed using the purified recombinant protein. Both proteins need a glucan polymer to act as a primer substrate and, initially maltodextrins were used with MalP and glycogen with GlgP as these have been reported to be the preferred primer substrate for both enzymes (Schwarz and Hofnung, 1967).

One problem that was identified whilst using maltodextrins as substrate was that the resulting product did not precipitate upon addition of ethanol. This is most likely because the enzyme did not extend the dextrin to a molecular size where it precipitates in 90% (v/v) ethanol, a hypothesis that is supported by the observation that the problem was not encountered when glycogen was used as a primer. This is a problem as the glucan synthesized by MalP needs to be separated from any remaining G1P and/or GBP prior to digestion as the phosphate attached to these sugars is acid labile. If the samples had been lyophilized, for example, both phosphate and glucose would be released from G1P and/or GBP during digestion and would interfere with the assay. However, the hypothesis being tested is whether or not G6P can be incorporated into glycogen (and not maltodextrins) by both phosphorylase enzymes. As MalP has been demonstrated to be capable of utilizing glycogen as a substrate (Becker *et al.*, 1994), it was decided to use this polyglucan in experiments using both MalP and GlgP. Additionally, results indicated possible sugar carry over and contamination, although this problem was negated by increasing the wash steps (Fig. 2.2). The sugar contamination was removed by increasing the number of wash steps from three to five.

Initial experiments were performed to examine if both enzymes could use GBP as a substrate and incorporate glucose 6-phosphate (G6P) into glycogen. It was found that G6P became incorporated when either enzyme was incubated with glycogen and either GBP alone, or a mixture of G1P and GBP (Fig. 2.3). No G6P was detected when the incubations were performed with only G1P. Interestingly, more G6P became incorporated when higher amounts of GBP were used in the reactions.

The incubation was optimized and performed with a series of substrate concentrations (Fig. 2.4). To determine the Km and V_{max} of MalP and GlgP when using

GBP, Lineweaver Burke graphs were plotted. The Km for GBP was calculated as approximately 1.3 mM for GlgP (Fig. 2.5, A) and 0.7 mM for MalP (Fig. 2.5, B). The V_{max} for GBP was determined to be approximately 0.08 µmol/µg/min for GlgP and 0.05 µmol/µg/min for MalP.

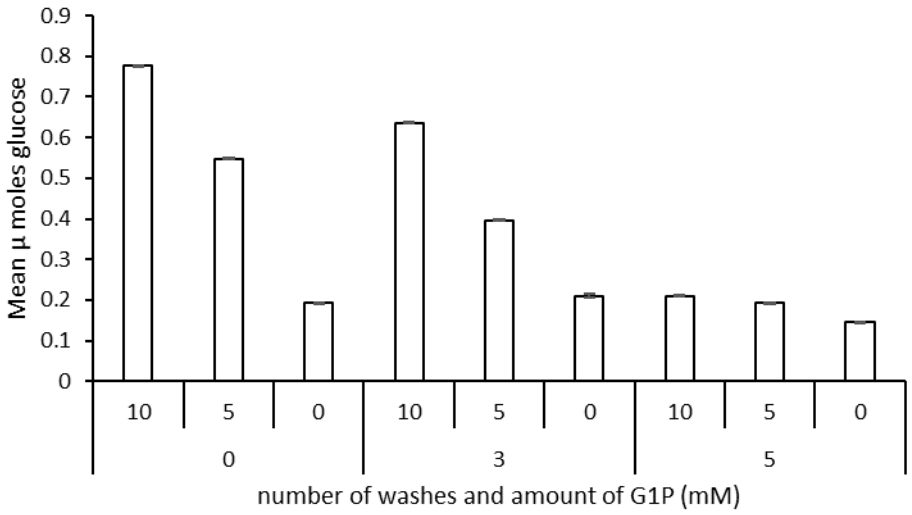

Figure 2.2 Glucose measurements following wash steps where glycogen and varying concentrations of G1P were precipitated and measured following zero, three, and five wash steps respectively, to ensure no sugar carry over. Data are presented as mean ± SE (n = 3).

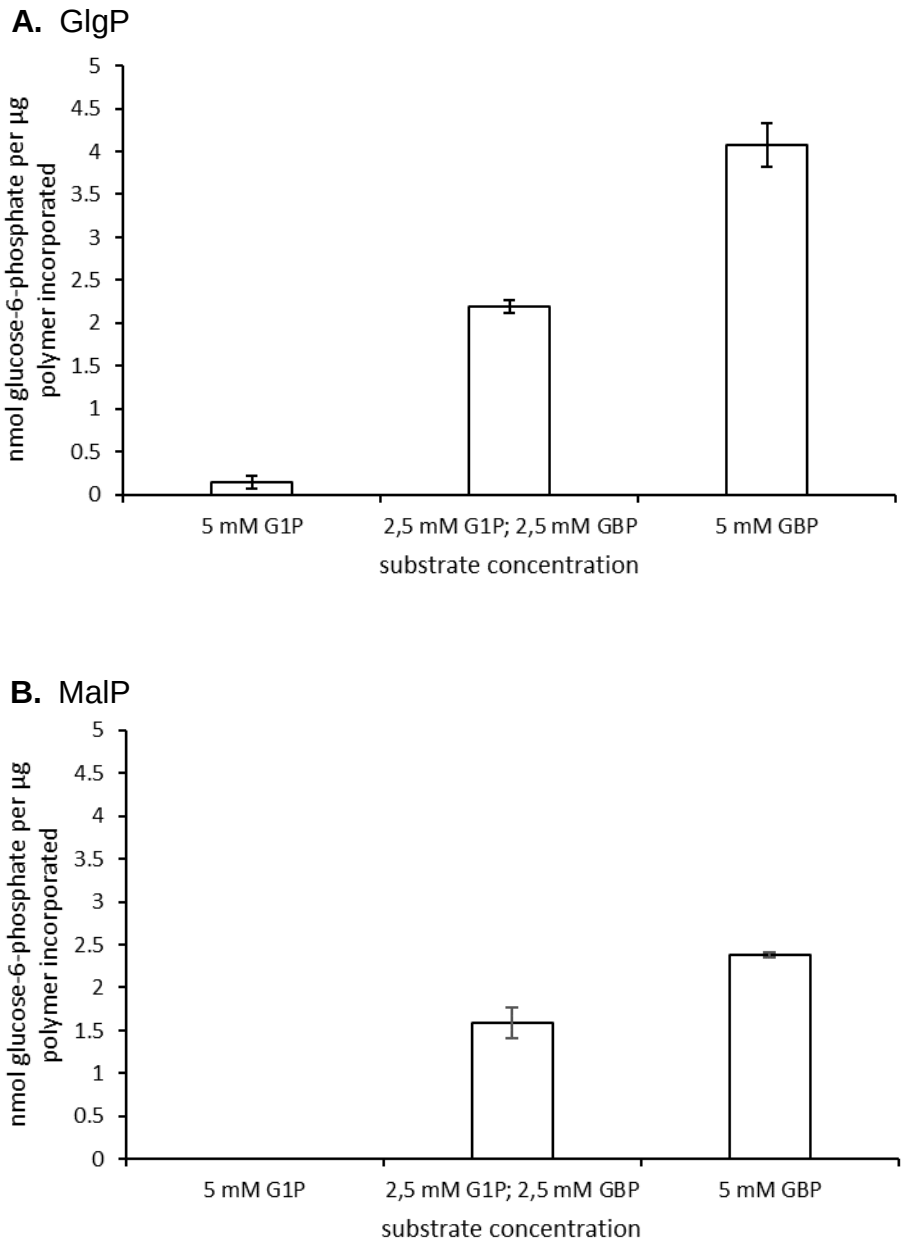

Figure 2.3 Phosphate incorporation into glycogen by phosphorylase enzymes. The recombinant protein was incubated with G1P and GBP in different ratios for 24 hours. The resultant nmol G6P incorporated per mg polymer is displayed as mean ± SE (n = 3). A, enzyme incubation with GlgP. B, enzyme incubation with MalP.

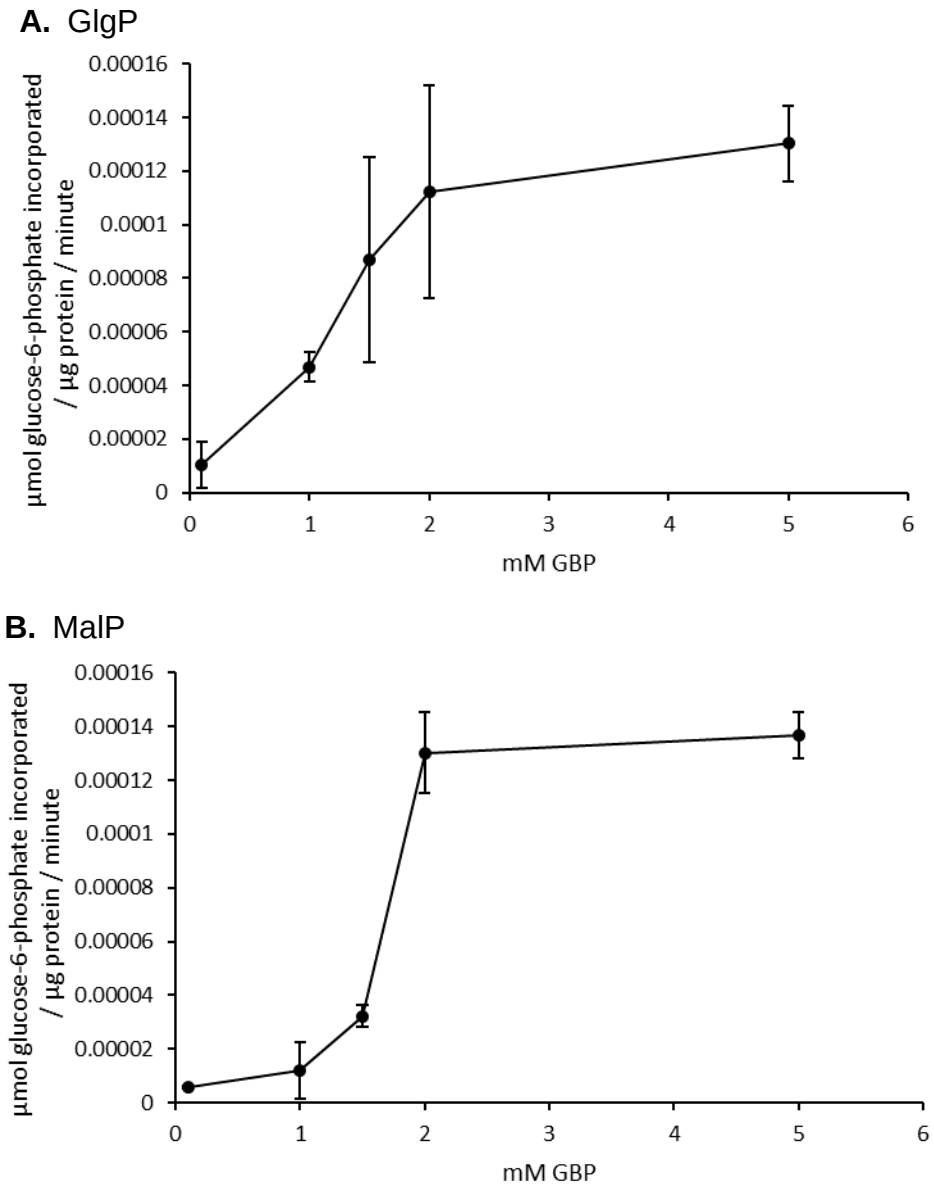

Figure 2.4 Phosphate incorporation per µg protein per minute for incubations with glycogen as a primer and varying concentrations of GBP as a substrate. Data are presented as mean ± SE (n=3). A, incubation with GlgP. B, incubation with MalP.

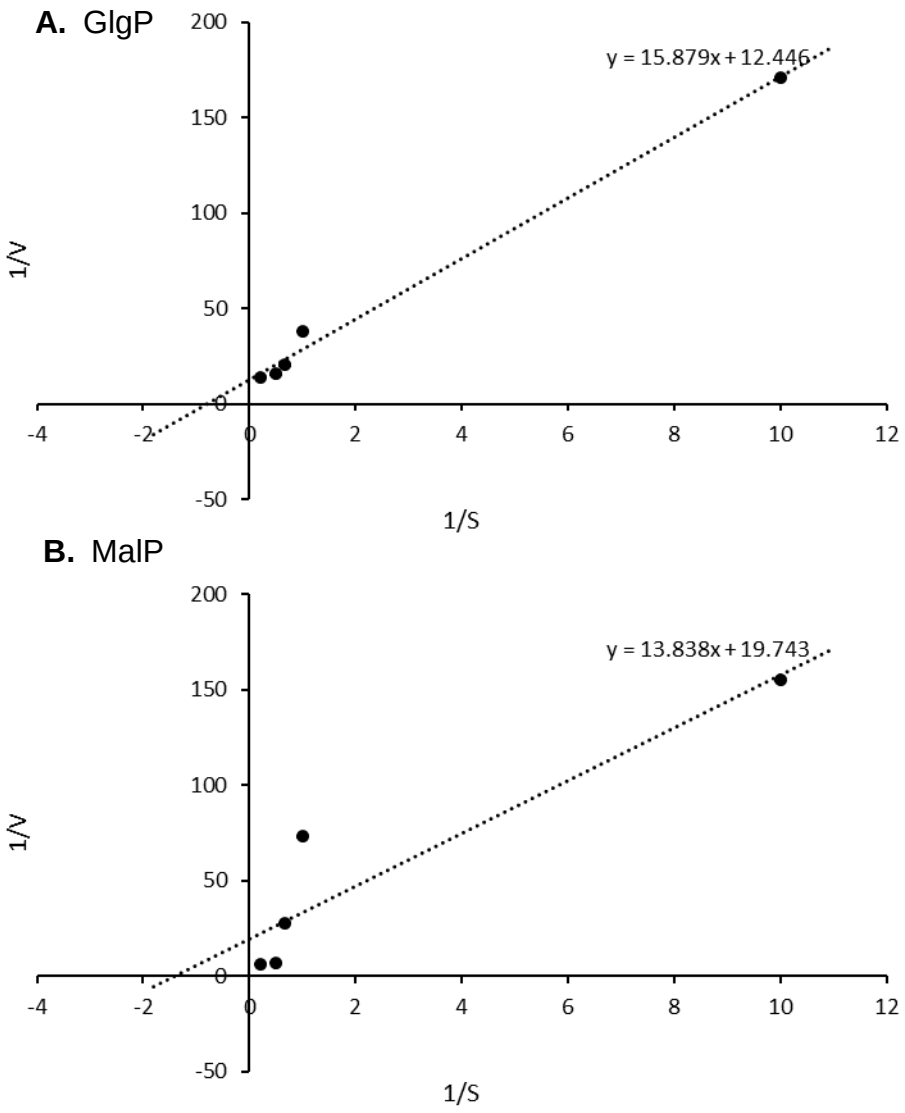

Figure 2.5 Lineweaver Burk Plots for phosphorylase enzymes incubated with varying concentrations of GBP as a substrate, and glycogen as a primer. The incubation was carried out over two hours at 37 °C. A, GlgP. B, MalP.

2.3.3 ^{31}P NMR analysis

MalP incubations were analyzed by ^{31}P NMR to examine the phosphate present within the polyglucan. Standards that were prepared included the various phosphorylated substrates used in the incubations as well as NAD that acts as a reference (Fig. 2.6). As expected, the presence of P_i corresponded to one signal peak at approximately 1 ppm. GBP presented two peaks at approximately 5.2 and 2.9 ppm, corresponding to the two phosphate monoesters present in the sugar. There was a third minor peak at 3 ppm. G6P and G1P presented peaks at approximately 4.6 and 3.2 ppm respectively, with G6P presenting a second minor peak at 4.4 ppm.

The results from the NMR analysis displayed no peaks when GlgP was used (data not shown). When MalP was incubated with GBP, a signal was detected approximately 0 and 2.25 ppm. When it was incubated with both G1P and GBP, two peaks were also detected, but at approximately -0.5 and 1.75 ppm. These appear to not be due to the enzyme as similar peaks were identified when the same experiment was performed with either no enzyme, or heat inactivated enzyme. (Fig. 2.7).

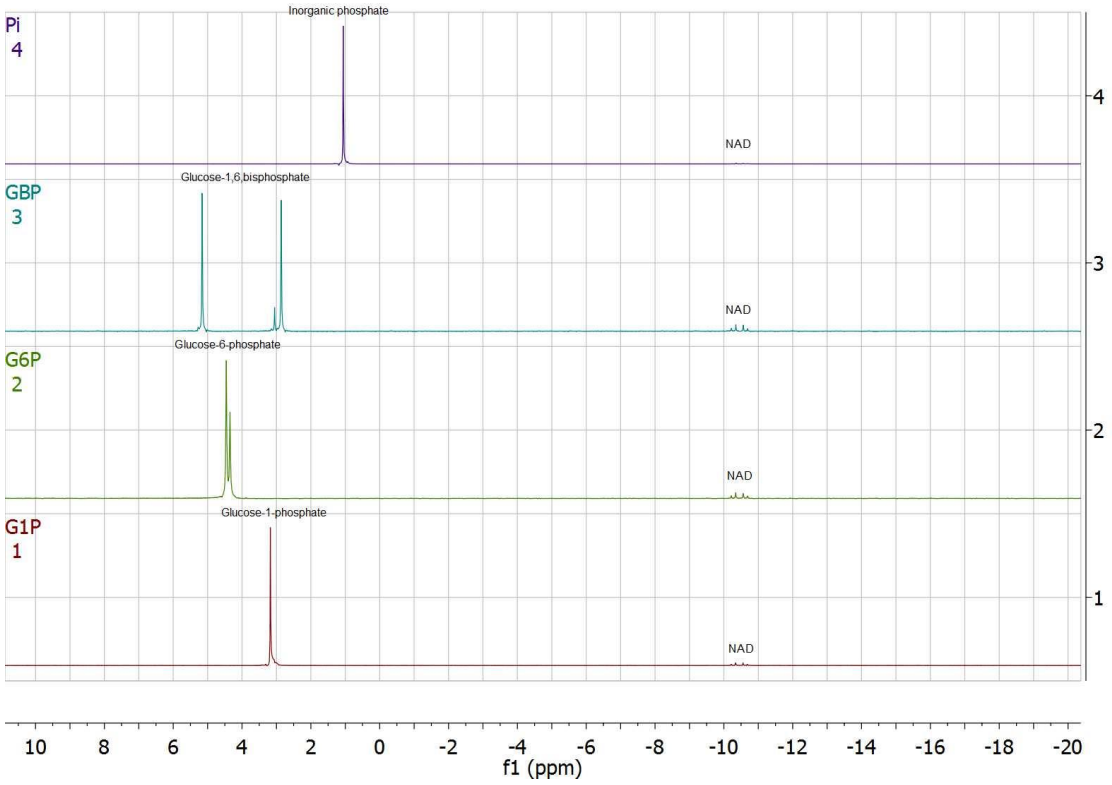

Figure 2.6 NMR standards analyzed at 300 MHz. Standards were at a concentration of 14 mg/mL, whilst the internal standard (NAD^+) was at a concentration of 1 mg/mL

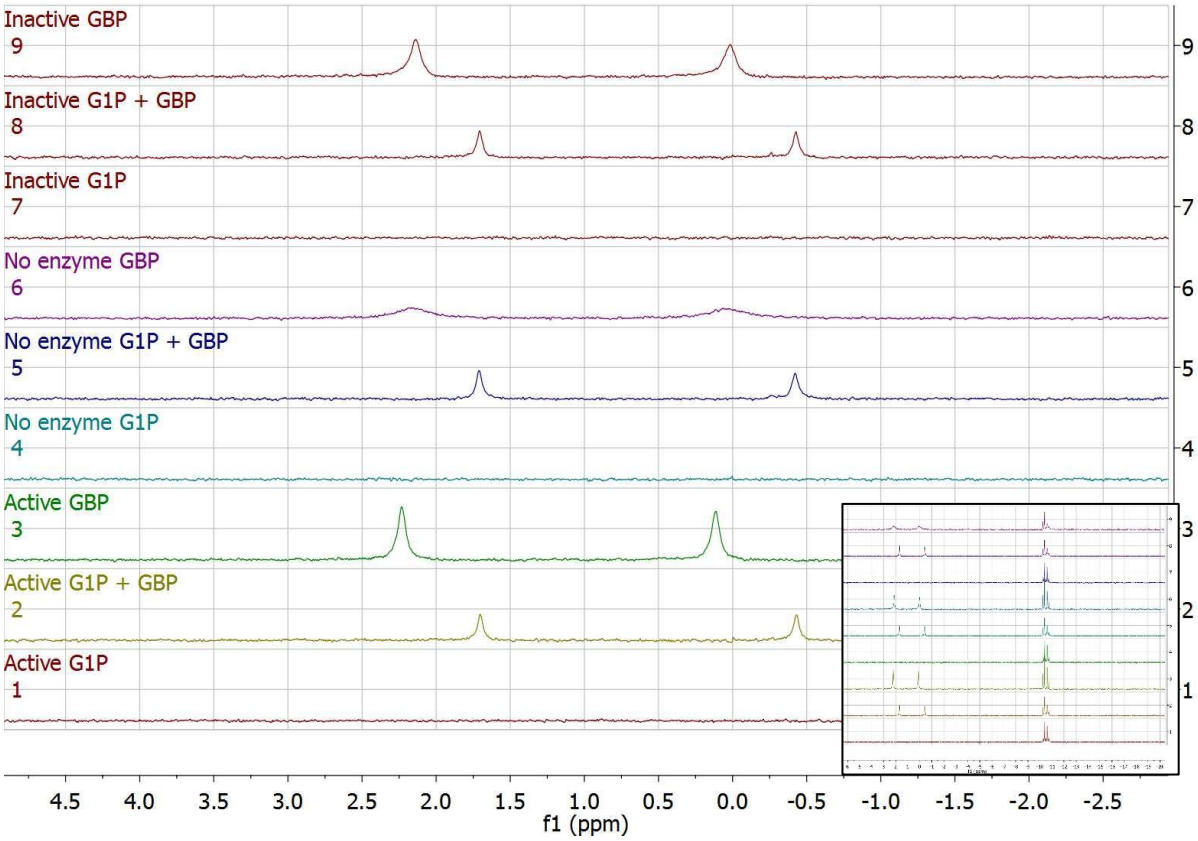

Figure 2.7 MalP incubation with either 5mM GBP, 5mM G1P and or 2.5mM G1P and GBP. Controls include incubations without protein, as well as incubations with inactive protein were all analyzed by NMR at 30 MHz under the same conditions. Insert: full scale with NAD^+ standards visible.

2.3.4 *In vivo* analysis

In order to test the hypothesis that GlgP and MalP have the ability to incorporate phosphate into glycogen *in vivo*, knock-out mutants lacking either GlgP and/or MalP (Strydom *et al.*, 2017) were cultured on solid Kornberg medium. Two independent strains for each of the single and double mutants were used. Glycogen was extracted from cells grown at 37°C overnight and digested to its constituent monomers by acid hydrolysis. The amounts of glucose and glucose-6-phosphate present were then determined and used to calculate the G6P content of the glycogen. It can be seen (Fig 2.8) that the phosphate content from glycogen extracted from the wild-type strain was approximately 1.2 nmol G6P/mg glycogen. Although one strain lacking MalP contained significantly reduced amounts of glycogen bound phosphate, the other MalP mutant strain did not and neither did any strain lacking GlgP or combinations of GlgP and MalP.

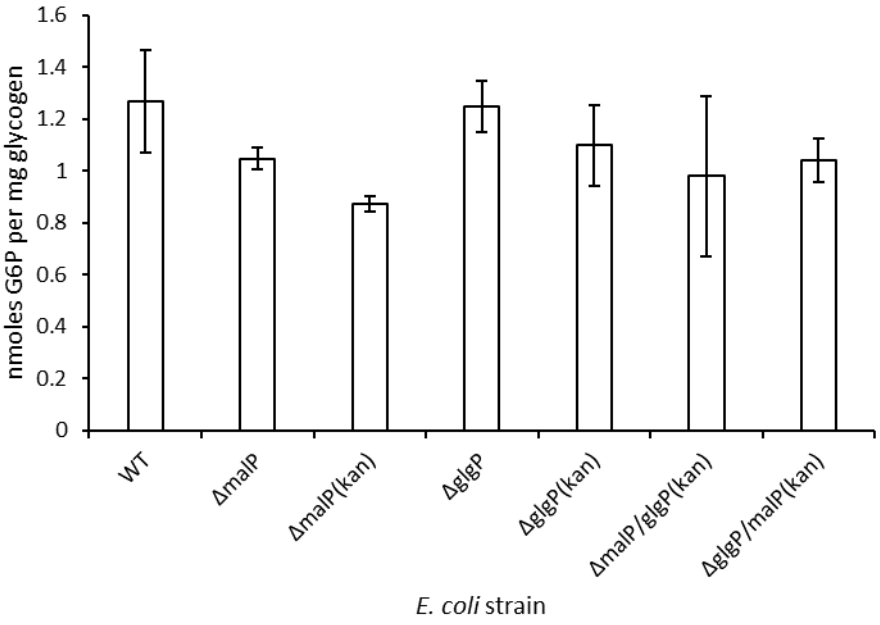

Figure 2.8 *In vivo* examination of phosphate content in glycogen extracted from knockout mutants of the GlgP and MalP enzymes. Data represented as mean ± SE (n=5). Tests for statistically significant differences were performed with a one-way ANOVA followed by a Bonferroni-Holm posthoc test.

2.4 Discussion

This study examined the role of two phosphorylases in *E. coli* glycogen phosphorylation. Both *in vitro* and *in vivo* analyses were performed to examine if they were capable of phosphorylating a growing polyglucan and to see if they perform this function during glycogen synthesis.

Firstly, GlgP and MalP were purified to homogeneity (Fig. 2.1 A, lanes 3 and 6). In the non-denaturing PAGE gel (Fig. 2.1 B), the lanes containing the empty vector controls show activity, which can be attributed to the native GlgP and MalP enzymes present in *E. coli*. When either GlgP or MalP expression was induced, there was an increase in production and activity of that specific protein. The GlgP showed different activity after purification, as the band was considerably lower than that in the crude extracts. GlgP may be bound to HPr (a component of the bacterial sugar phosphotransferase transport system known to interact with GlgP (Seok *et al.*, 2001)) in the crude extract, which would be removed during the purification process, resulting in GlgP activity being visualized lower in the gel for the pure protein. Irrespective of this, both enzymes were active and could be used to test the theory that they are able to phosphorylate glycogen.

An experiment with both G1P and GBP was therefore set up to test this theory. After optimizing the experimental setup to ensure reliability of results (Fig. 2.2), it was found that both GlgP and MalP were able to incorporate phosphate into glycogen in the form of G6P using GBP as a substrate (Fig. 2.3). It has been shown previously that MalP has a lower affinity for larger glucans (such as glycogen) than for smaller maltodextrins (Schwarz and Hofnung, 1967), while GlgP has a higher affinity for glycogen (Chen and Segel, 1968a; 1968b). Despite this, both enzymes were able to extend glycogen by adding both glucose and glucose-6-phosphate monomers to glycogen primers during the incubation, demonstrated by the polymer forming activity, as well as the presence of G6P in polymers after incubation. MalP and GlgP have both been found to have a K_m of approximately 1 mM for G1P (Chen and Segel, 1968b; Becker *et al.*, 1994; Boeck and Schinzel, 1996). The K_m was determined after the enzymes were incubated with varying concentrations of GBP (Fig. 2.4) and a double-reciprocal plot was produced (Fig. 2.5). The K_m for GBP incorporation was experimentally determined to be very similar, at ~1.3 mM for GlgP, and ~0.7 mM for

MalP, whilst the V_{max} for GBP was ~0.08 µmol/µg/min for GlgP, and ~0.05 for MalP µmol/µg/min.

Analysis of *in vitro* synthesized polyglucans to examine glucose 6-phosphate amounts by ^{31}P NMR was inconclusive. This technique has been used successfully in the past to examine phosphate covalently bound to starch and the signal of G6P when present in a polyglucan is observed at approximately 4 ppm (Kasemsuwan and Jane, 1996). Unfortunately, the peaks observed in this experiment were also present in the negative controls (Fig. 2.7) and do not correlate with the P_i, G1P, G6P or GBP standards (Fig. 2.6). A possible explanation for this is the lack of a buffer included in the sample, since changes in pH can result in shifted peaks. This will be optimized and repeated for both MalP and GlgP, however, due to time constraints, data cannot be included in this study.

The results of this part of the thesis confirm the hypothesis that MalP and GlgP are able to incorporate phosphate into polymers in the form of G6P when they utilize GBP as a substrate. This suggests that the mechanism of *in vitro* phosphate incorporation into glycogen by *E. coli* may be catalyzed by these two phosphorylase enzymes and relies on the presence of GBP. When catalyzed by MalP and GlgP, incorporation of the glucose moiety through cleavage of the phosphate on C-1 results in the incorporation of a phosphorylated moiety, thereby increasing the phosphate content of the polyglucan in question. This is the first time it has been demonstrated that phosphorylase enzymes can incorporate phosphate into glycogen. However, it does not show that this occurs *in vivo*. For that, analysis of glycogen from mutants lacking GlgP and MalP would have to be examined. Such strains have recently been manufactured and used to study glycogen accumulation in *E. coli* (Strydom *et al.*, 2017). In that study, it was demonstrated that the double mutant lacking both GlgP and MalP accumulate more glycogen than the single mutants, which in turn accumulate more than the wild-type control strain. However, glycogen phosphate was not determined.

To examine the amount of phosphate present in the glycogen of the strains, they were transformed with the plasmid pACAG (Kossmann *et al.*, 1999), which increases the amount of glycogen accumulated due to the expression of an unregulated ADP-glucose pyrophosphorylase encoded by the *glgc16* gene (Creuzat-

Sigal *et al.*, 1972). The *E. coli* strains were also grown on solid media rather than liquid media, as this has been found to result in greater glycogen accumulation (Jewell, 2015). Cells from 18 plates with an approximate surface area of 500 cm^2 were collected for each of the seven *E. coli* strains. Glycogen was then extracted from the cells for each strain and was then acid digested and G6P content determined. It established that glycogen from all of the strains contains similar amounts of G6P (Fig. 2.8). This indicates that although GlgP and MalP can phosphorylate glycogen *in vitro*, they do not appear to do so *in vivo* in *E. coli*. GBP is present in incredibly low amounts in biological systems. Whilst G1P is normally thought to be present in the mM range (Bennett *et al.*, 2009), GBP is present only in the nM range (Beitner, 1984; Sicher and Kremer, 1990). Given that the K_m for GBP and G1P are similar, and that the amount of GBP present is approximately 1000x lower than that of G1P (as would be expected), it would be anticipated that GlgP and/or MalP are incorporating 1000x less G6P than glucose. Bearing that in mind, we would expect to see less G6P in glycogen that we do if it were incorporated by GlgP and MalP *in vivo*. Additionally, the majority of the glucose moieties are incorporated by GlgA, so the small amount potentially incorporated by GlgP and MalP would have to be almost all G6P to be detected. This would only be possible if the K_m for GBP was an order of magnitude lower than the K_m for G1P, indicating that it would preferentially use GBP over G1P. However, considering the enzymes show similar K_m for G1P and GBP, they would incorporate the two moieties in a manner proportional to the substrate availability. As mentioned earlier, this is far lower for GBP than G1P (Bennett *et al.*, 2009; Beitner, 1984; Sicher and Kremer, 1990). The data indicate that GlgP and MalP are not the enzymes responsible for *in vivo* phosphate incorporation in *E. coli*.

This study has therefore shown that both enzymes can incorporate G6P *in vitro*, however, they do not do so *in vivo*. Future work will focus on elucidation of the mechanisms by which glycogen is phosphorylated *in vivo* in *E. coli*. There is no obvious GWD ortholog in the *E. coli* genome, so it would be worthwhile investigating whether GlgA incorporated phosphate into bacterial glycogen, as it is hypothesized to in mammals. This will be the subject of future work.

Chapter 3: General discussion

Phosphate has been shown to be present in both starch and glycogen, two similar storage polymers found in plants, and bacteria and mammals. Although the mechanism whereby phosphate is incorporated into starch is well understood, glycogen phosphorylation is not well understood, particularly within bacteria. Glycogen synthases have been hypothesized to phosphorylate mammalian glycogen (Tagliabracci *et al.*, 2011), and Contreras *et al.* (2016) found that it is capable of doing so *in vitro* (with the β-phosphate of UDP-glucose acting as a phosphate donor), but not *in vivo*. The presence of phosphate in glycogen from wild type *E. coli* (Lorberth *et al.*, 1998; Viksø-Nielsen *et al*. 2002) suggests that the components for phosphorylation of glycogen must be present in bacteria, and this project provides insight into the process whereby this occurs.

This project was designed to examine the potential roles of GlgP and MalP in the incorporation of phosphate into glycogen. There is evidence of the phosphate being incorporated into polyglucans by MalP and GlgP *in vitro*, whereby G6P is incorporated into the polymer when GBP is used as a substrate. However, *in vivo* analyses for both enzymes indicated that this does not occur *in vivo*. This indicates that there is another pathway whereby phosphate is incorporated into glycogen in *E. coli*, as there was phosphate present in the glycogen isolated from WT and all mutant strains, confirming that it is incorporated into the polymer in bacteria. This will be studied further in future.

Despite the lack of evidence that GlgP and MalP incorporated phosphate into glycogen, the similarities between the glycogen and starch biosynthetic pathways means that it may be able to do this in other systems, such as starch (Viksø-Nielsen *et al.*, 2002). These findings could potentially be applied in biotechnological fields, whereby starch could be modified to be more suitable for industrial uses. There would be a number of potential difficulties in doing this. Firstly, GBP quantities in the plasmid would need to be increased, as it is only found in nM concentrations in plants (Sicher and Kremer, 1990). GBP producing enzymes, such as the mammalian glucose-1,6-bisphosphate synthase (Maliekal *et al.*, 2007), or PgcM, a bacterial β-phosphoglucomutase producing GBP as an intermediate substrate (Mesak and Dahl, 2000) could potentially be expressed in the plastid to increase GBP production. This

would, in turn, increase the amount of GBP available as a substrate, allowing more phosphate to be incorporated into the polyglucan. Additionally, the K_m for GBP could be improved through directed evolution, a powerful tool to improve the catalytic activity of an enzyme (Li *et al.*, 2017).

In conclusion, this study confirmed the ability of GlgP and MalP to incorporate phosphate in glycogen *in vitro*, although they did not demonstrate this activity *in vivo*. Future work will involve adapting the system to produce highly phosphorylated starch for utilization in industry.

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